JOURNAL OF BACTERIOLOGY, Oct. 1967, p.1046-1051

Vol. 94, No. 4
Printed in U.S.A.

Mechanism of Bacterial Pyrite Oxidation

MELVIN P. SILVERMAN[1]

Pittsburgh Coal Research Center, Bureau of Mines, U.S. Department of the Interior, Pittsburgh, Pennsylvania 15213

Received for publication 5 July 1967

The oxidation by *Ferrobacillus ferrooxidans* of untreated pyrite (FeS_2) as well as HCl-pretreated pyrite (from which most of the acid-soluble iron species were removed) was studied manometrically. Oxygen uptake was linear during bacterial oxidation of untreated pyrite, whereas with HCl-pretreated pyrite both a decrease in oxygen uptake at 2 hr and nonlinear oxygen consumption were observed. Ferric sulfate added to HCl-pretreated pyrite restored approximately two-thirds of the decrease in total bacterial oxygen uptake and caused oxygen uptake to revert to nearly linear kinetics. Ferric sulfate also oxidized pyrite in the absence of bacteria and O_2; recovery of ferric and ferrous ions was in excellent agreement with the reaction $Fe_2(SO_4)_3 + FeS_2 = 3FeSO_4 + 2S$, but the elemental sulfur produced was negligible. Neither H_2S nor $S_2O_3^{2-}$ was a product of the reaction. It is probable that two mechanisms of bacterial pyrite oxidation operate concurrently: the direct contact mechanism which requires physical contact between bacteria and pyrite particles for biological pyrite oxidation, and the indirect contact mechanism according to which the bacteria oxidize ferrous ions to the ferric state, thereby regenerating the ferric ions required for chemical oxidation of pyrite.

The rate of oxidation of a number of sulfide minerals is markedly accelerated by the acidophilic, chemoautotrophic, iron-oxidizing bacteria *Thiobacillus ferrooxidans*, *Ferrobacillus ferrooxidans*, and related species. These bacteria play important roles in geochemical mineral transformations, in problems of water pollution associated with acid mine drainage, in the leaching and recovery of valuable metals from metal sulfide ores, and in eliminating pyritic sulfur from coal (7, 10, 14, 20).

The mechanism of bacterial attack on sulfide minerals is not fully understood. Two different mechanisms have been proposed (14) which, for convenience, will be referred to as the indirect contact and direct contact mechanisms. According to the indirect contact mechanism, ferric ions are the primary oxidant, oxidizing metal sulfides while being reduced in turn to the ferrous state. The bacteria then enter the reaction by oxidizing ferrous ions to the ferric state, thereby regenerating the primary oxidant. The direct contact mechanism is independent of the action of ferric ions, requiring only intimate physical contact between the bacteria and sulfide mineral under aerobic conditions. Operation of the direct contact mechanism is suggested by observations of bacterial acceleration of the rate of oxidation of iron-free sulfide minerals such as covellite (CuS), chalcocite (Cu_2S), tetrahedrite ($Cu_8Sb_2S_7$), molybdenite (MoS_2), orpiment (As_2S_3), and synthetic CuS and Cu_2S in essentially iron-free systems (2-5, 11, 12). This mechanism becomes difficult to demonstrate with iron-containing sulfide minerals such as arsenopyrite ($FeS_2 \cdot FeAs_2$), bornite (Cu_5FeS_4), and chalcopyrite ($CuFeS_2$), owing to the release of soluble iron during oxidation and probable concurrent operation of the indirect contact mechanism.

The mechanisms of bacterial pyrite oxidation have been investigated in connection with our research on the bacterial removal of pyritic sulfur from coal (18). The present paper presents the results of studies aimed at determining whether either or both of the above mechanisms take place during the bacterial oxidation of pyrite.

MATERIALS AND METHODS

Media and cultures. *F. ferrooxidans* was grown autotrophically in ferrous sulfate-mineral salts medium 9K, harvested, washed, and resuspended in dilute H_2SO_4 (*p*H 3.5) as previously described (15).

Pyritic material. Pyrite samples 29 (77.0%, pyrite) and 30 (60.0%, pyrite) were the same as used in an earlier study (17) and consisted of pyrite concentrates from raw coals whose original pyrite contents were 4.5 and 8.2%, respectively. Both samples were ground

[1] Present address: National Aeronautics and Space Administration, Ames Research Center, Moffett Field, Calif. 94035.

to pass through a 325-mesh screen. Acid-pretreated samples were prepared by refluxing the pyrite in 2 N HCl for 45 min on a steam bath, washing thoroughly with distilled water until no chloride could be detected with $AgNO_3$, and drying overnight in a vacuum oven. Acid-pretreated samples were stored under N_2.

Analytical method. Total soluble iron (ferrous and ferric) was determined by the colorimetric American Society for Testing Materials *o*-phenanthroline method. Ferrous iron was selectively determined in mixtures of Fe^{++} and Fe^{3+} by omitting the reducing agent from the procedure. Ferric iron was determined by difference (total iron − ferrous = ferric). A standard curve over the concentration range 20 to 240 μg of iron was prepared, reading optical density at 500 mμ in a Bausch & Lomb Spectronic-20 colorimeter.

The acid-soluble iron of pyrites was determined immediately before use. Iron was extracted by refluxing the material in 2 N HCl for 30 min on a steam bath, filtering through Whatman no. 40 or Schleicher and Schuell no. 589 blue ribbon filter paper, followed by several distilled water rinses. Filtrate and rinse water were combined, brought to a volume of 50 ml with distilled water, and suitable samples were taken for the colorimetric iron determinations. The acid-soluble iron in cell suspensions (bacterial iron) was determined similarly. Bacterial nitrogen was determined by a micro-Kjeldahl procedure.

Manometric procedure. Oxygen uptake was measured manometrically with the Warburg respirometer in the conventional manner (21). Each Warburg flask contained 20 mg of pyritic material and 1.0 ml of dilute H_2SO_4 (*p*H 3.5) in the main compartment, 0.5 ml of cell suspension in the side arm, and 0.2 ml of 20% (w/v) KOH plus a 2-cm square of Whatman no. 42 filter paper in the center well. Neither autoxidation of pyrites nor endogenous metabolism of "resting cells" could be detected. Ferric ion solutions were prepared by dissolving $FeCl_3 \cdot 6H_2O$ or $Fe_2(SO_4)_3 \cdot {}_nH_2O$ in dilute H_2SO_4 (*p*H 2.6).

RESULTS

In previous manometric studies (17), a linear rate of oxygen uptake was observed during oxidation of a number of different pyrite samples by "resting cells" of *F. ferrooxidans*. One exception was a $CaCO_3$-containing pyrite which resisted oxidation. Upon pretreatment with HCl, which removed acid-soluble iron species as well as carbonates, this pyrite became susceptible to bacterial attack. However, oxygen uptake was nonlinear and was preceded by a 2-hr lag period. This finding prompted further studies on the role of acid-soluble iron in bacterial pyrite oxidation.

Hydrochloric acid pretreatment removed most of the acid-soluble iron from pyrites 29 and 30 (Table 1). Figure 1 shows that the rates of oxygen uptake during bacterial oxidation of untreated pyrites were linear in contrast to the nonlinear uptake of oxygen with acid-pretreated pyrites. The effect was more pronounced with pyrite 30 than with 29. Pyrite 29 was previously found to be more susceptible to bacterial oxidation than pyrite 30 (17). Removal of the acid-soluble iron associated with the pyrites did not alter their relative rates of oxidation.

TABLE 1. *Effect of hydrochloric acid pretreatment on the HCl-soluble iron content of pyrites 29 and 30*

Pyrite	HCl-soluble iron (μg/20 mg of pyrite)	
	Found	Avg
29, untreated	1,328 1,208	1,268
29, pretreated	56 70	63
30, untreated	704 688	696
30, pretreated	36 32	34

The question arose whether the effect of removal of acid-soluble iron by acid pretreatment could be reversed by restoring the soluble iron content to its original level. Figure 2 shows that the addition of 633 μg of Fe^{3+} (as $FeCl_3$) was slightly inhibitory, whereas 1,266 μg of Fe^{3+} (as $FeCl_3$) completely inhibited the bacterial oxidation of acid-pretreated pyrite 30. Neither *F. ferrooxidans* (16) nor *T. ferrooxidans* (1, 8) is inhibited by high concentrations of ferrous or ferric ions. The inhibition can only be attributed to the chloride moiety, in agreement with earlier reports of chloride inhibition of *T. ferrooxidans* (8, 9, 12). When ferric sulfate was employed, however, ferric ions succeeded in reversing the effect of acid pretreatment on the bacterial oxidation of pyrite 30 (Fig. 3). Ferric ions at 130 or 260 μg were equally effective in restoring the rate of pyrite oxidation by about two-thirds and caused oxygen uptake to revert to nearly linear kinetics.

These results (Fig. 1 and 3) suggested that ferric iron is a participant in the bacterial oxidation of pyrite. Stokes (19) postulated that the following chemical reactions might take place:

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2S \quad (1)$$

$$2S + 6Fe_2(SO_4)_3 + 8H_2O \rightarrow 12FeSO_4 + 8H_2SO_4 \quad (2)$$

$$\text{Sum: } FeS_2 + 7Fe_2(SO_4)_3 + 8H_2O \rightarrow 15FeSO_4 + 8H_2SO_4 \quad (3)$$

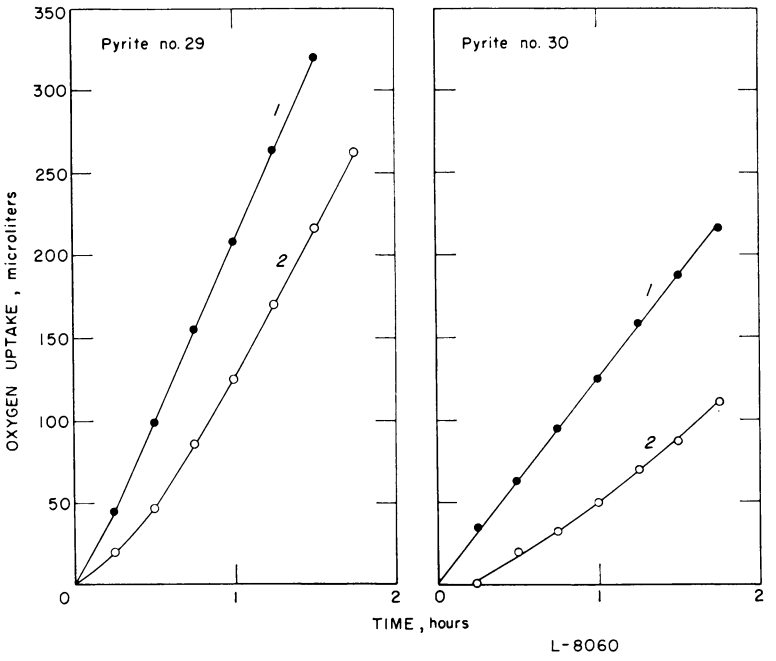

FIG. 1. *Oxidation of HCl-pretreated pyrites 29 and 30 by Ferrobacillus ferrooxidans. Curve 1, untreated pyrite; curve 2, acid-pretreated pyrite. Initial pH 3.5; bacterial nitrogen, 0.16 μg; and bacterial iron, 34 μg for all flasks.*

Leathen (10) suggested that ferric sulfate oxidized pyrite to ferrous sulfate while being reduced in turn to the ferrous state, and that the role of *F. ferrooxidans* in pyrite oxidation was to regenerate ferric ions. Temple and Koehler (20) reported that ferric sulfate accelerated the release of soluble iron from an aqueous suspension of pulverized pyrite. Garrels and Thompson (6) found that at *p*H 0 to 2 the oxidation of pyrite by ferric sulfate under N_2 was in overall agreement with reaction 3.

The oxidation of pyrite by Fe^{3+} was examined in a system in which both oxygen and bacteria were excluded. Acid-pretreated pyrite 29 (100 mg) was added to each of three 20 by 155 mm rubber-stoppered test tubes. The control tube received 10 ml of dilute H_2SO_4 (*p*H 2.6). Duplicate experimental tubes received 8 ml of dilute H_2SO_4 and 2 ml of acid ferric sulfate solution (653 μg of Fe^{3+}). Nitrogen, washed with alkaline pyrogallol to remove traces of O_2, was bubbled continuously through all tubes. After 3 days, the contents were analyzed for HCl-soluble ferrous and ferric iron. The results of this experiment (Table 2) demonstrate that ferric ions oxidize pyrite in the absence of O_2 and bacteria. The quantities of Fe^{3+} consumed and Fe^{2+} produced are in excellent agreement with the quantities predicted by reaction 1.

To determine whether elemental sulfur was in fact a product of reaction 1, 45 ml of acid ferric sulfate solution (63.4 mmoles of Fe^{3+}; *p*H 2.6) and 5 g of acid-pretreated pyrite 29 were added to a rubber-stoppered 100-ml Pyrex centrifuge bottle. The control bottle contained 45 ml of dilute H_2SO_4 (*p*H 2.6) in place of the ferric sulfate solution. Oxygen-free N_2 (obtained by passage over hot copper) was bubbled in series through distilled water humidifiers, the pyrite mixtures, and 5% cadmium acetate solutions (H_2S trap). After 8 days at room temperature, at which time no precipitate was detected in the H_2S traps, the solids were collected in Soxhlet thimbles, dried overnight in a vacuum oven at 50 C, and extracted with CCl_4 for 7 hr under N_2 in a Soxhlet apparatus. The CCl_4 was evaporated under N_2 and the dry residue was weighed and analyzed for elemental sulfur. The results (Table 3) show that the amount of elemental sulfur produced was far below expectation, being only 3.5% of the amount required by reaction 1.

DISCUSSION

The results of the manometric experiments suggest concurrent operation of both the direct contact and indirect contact mechanisms. The following experimental observations support the indirect contact mechanism. (i) Removal of

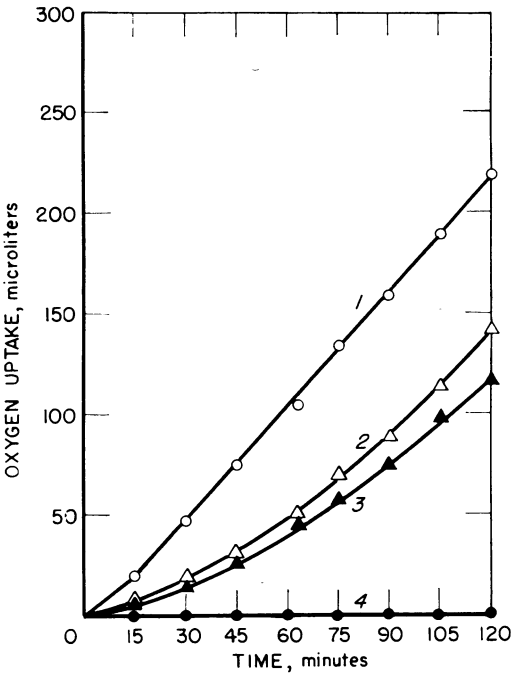

FIG. 2. *Effect of ferric chloride on the oxidation of acid-pretreated pyrite 30 by Ferrobacillus ferrooxidans. Curve 1, untreated pyrite, initial pH 3.5. Curve 2, acid-pretreated pyrite, initial pH 3.5. Curve 3, acid-pretreated pyrite + 633 μg of Fe^{3+}, initial pH 3.65. Curve 4, acid-pretreated pyrite + 1,266 μg of Fe^{3+}, initial pH 3.7. Bacterial nitrogen, 0.16 μg, and bacterial iron, 34 μg for all flasks.*

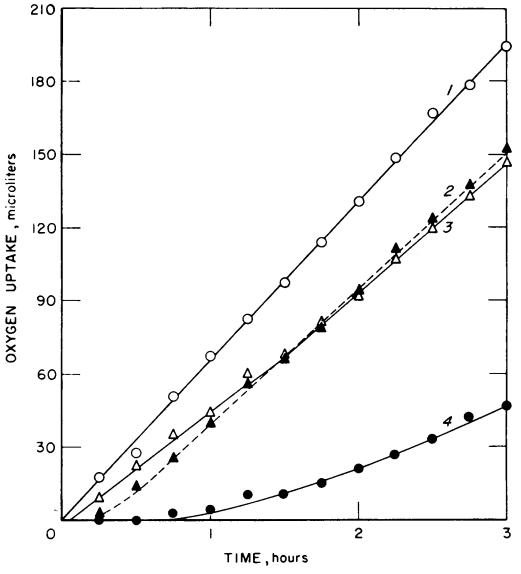

FIG. 3. *Effect of ferric sulfate on the oxidation of acid-pretreated pyrite 30 by Ferrobacillus ferrooxidans. Curve 1, untreated pyrite. Curve 2, acid-pretreated pyrite + 260 μg of Fe^{3+}. Curve 3, acid-pretreated pyrite + 130 μg of Fe^{3+}. Curve 4, acid-pretreated pyrite. Bacterial nitrogen, 0.16 μg, and bacterial iron, 34 μg; initial pH 3.5 for all flasks.*

acid-soluble iron from pyrite depressed the rate of bacterial pyrite oxidation while altering the rate of oxygen uptake from linear to nonlinear (Fig. 1 and 2, curves 1 and 2; Fig. 3, curves 1 and 4). (ii) Addition of ferric ions as sulfates to pyrite restored the rate of oxygen uptake by about two-thirds, virtually eliminated the lag period, and caused the rate of oxygen uptake to become nearly linear (Fig. 3). Chemical oxidation of pyrite by ferric ions may be the rate-limiting step in the overall rate of the indirect contact mechanism. This is suggested by the failure of 260 μg of Fe^{3+} to increase further the rate of oxidation over the rate obtained with 130 μg of Fe^{3+}, assuming the ferrous ions produced (the bacterial substrate) were not in excess. The possibility that some chloride ions from the HCl pretreatment were adsorbed to pyrite surfaces and blocked some oxidizable sites might also account for the failure to obtain more than two-thirds restoration of oxygen uptake upon subsequent addition of ferric ions.

On the other hand, operation of a direct contact mechanism would necessarily require physical contact between insoluble pyrite particles and bacteria prior to detectable oxygen uptake. The total number of attachments between bacteria and acid-pretreated pyrite particles would be expected to increase with time and be reflected by an increasing rate of oxygen uptake, as shown in Fig. 1 and 3. With untreated pyrite, oxygen uptake during bacterial oxidation of the ferrous ions associated with this material could be superimposed on the increasing rate of oxygen uptake stemming from direct contact oxidation. The result would be the overall linear kinetics observed in Fig. 1 and 3. Nevertheless, other experimental evidence demonstrating a correlation between rates of bacterial pyrite oxidation and bacterial attachment to pyrite particles is needed to support further the direct contact mechanism.

The results in Table 2 show that ferric ions oxidize pyrite in the absence of oxygen and bacteria. Recovery of the iron species involved is in excellent agreement with reaction 1. However, the fate of sulfur in the reaction remains to be clarified. Hydrogen sulfide was not produced. Thiosulfate, if formed, would have signaled its presence by decomposing to elemental sulfur at the low *p*H (2.6) of the reaction mixture.

TABLE 2. *Oxidation of pyrite 29 by ferric sulfate*

Tube no.	Forms of iron	HCl-soluble iron (μg)					Per cent of theory
		At 0 days	At 3 days	Net change[a]			
				Theory[b]	Found		
					Uncorrected	Corrected[c]	
Control	Fe^{2+}	125	150		+25		
	Fe^{3+}	5	5		0		
	Total	130	155		+25		
1	Fe^{2+}	125	1,120	+987	+995	+970	98.3
	Fe^{3+}	658[d]	0		−658		
	Total	783	1,120		+337		
2	Fe^{2+}	125	1,080	+957	+955	+930	97.2
	Fe^{3+}	658[d]	20		−638		
	Total	783	1,100		+317		

[a] Plus sign = increase, minus sign = decrease.
[b] Calculated from Fe^{3+} actually consumed, based on reaction 1.
[c] Corrected for Fe^{2+} production in control.
[d] Includes 653 μg of Fe^{3+} added as $Fe_2(SO_4)_3$.

TABLE 3. *Formation of elemental sulfur during oxidation of pyrite 29 by ferric sulfate*

Bottle	Fe^{3+} added	Fe^{3+} reacted	Elemental sulfur	
			Expected	Found[a]
	mmoles	*mmoles*	*mmoles*	*mmoles*
Control.....	0		0	0.43
Experimental...	63.4	35.9	35.9	1.24

[a] Identified as elemental sulfur by X-ray diffraction and mass spectrometry.

Although the quantity of elemental sulfur found (Table 3) was far below the amount required by reaction 1, it is conceivable that elemental sulfur or some form of zero-valence sulfur other than the stable eight-membered cyclic structure was produced but oxidized rapidly to sulfate upon exposure to air during the operations immediately preceding the Soxhlet extraction. Alternatively, it may be that elemental sulfur as such was not a product of reaction 1. Sato (13) presented evidence for the oxidation of several sulfide minerals by "a process in which metal atoms move into the surrounding solution to become aqueous cations, accompanied by a stepwise decrease in the metal to sulfur ratio of the remaining solid phase." Oxidation of crystalline pyrite, Fe_nS_{2n}, may have resulted in the release of ferrous ions with the formation of a residual crystalline material, $Fe_{n-x}S_{2n}$, which retained its sulfur and was either insoluble in CCl_4 or was oxidized to sulfate upon momentary exposure to air.

ACKNOWLEDGMENTS

I thank E. Bean and A. G. Sharkey, Jr., for X-ray diffraction and mass spectrometric analyses.

LITERATURE CITED

1. BECK, J. V., AND F. M. SHAFIA. 1964. Effect of phosphate ion and 2,4-dinitrophenol on the activity of intact cells of *Thiobacillus ferrooxidans*. J. Bacteriol. **88**:850–857.
2. BRYNER, L. C., AND R. ANDERSON. 1957. Microorganisms in leaching sulfide minerals. Ind. Eng. Chem. **49**:1721–1724.
3. BRYNER, L. C., J. V. BECK, D. B. DAVIS, AND D. G. WILSON. 1954. Microorganisms in leaching sulfide minerals. Ind. Eng. Chem. **46**:2587–2592.
4. EHRLICH, H. L. 1962. Microbial association with some mineral sulfides, p. 153–168. *In* M. L. Jensen [ed.], Biogeochemistry of sulfur isotopes. National Science Foundation Symposium, Yale University, New Haven, Conn.
5. EHRLICH, H. L. 1963. Bacterial action on orpiment. Econ. Geol. **58**:991–994.
6. GARRELS, R. M., AND M. E. THOMPSON. 1960. Oxidation of pyrite by iron sulfate solutions. Am. J. Sci. **258**:57–67.
7. KUZNETSOV, S. I., M. V. IVANOV, AND N. N. LYALIKOVA. 1963. Introduction to geological microbiology. McGraw-Hill Book Co., Inc., New York.
8. LANDESMAN, J., D. W. DUNCAN, AND C. C. WALDEN. 1966. Iron oxidation by washed cell suspensions of the chemoautotroph, *Thio-*

bacillus ferrooxidans. Can. J. Microbiol. **12**: 25–33.

9. LAZAROFF, N. 1963. Sulfate requirement for iron oxidation by *Thiobacillus ferrooxidans*. J. Bacteriol. **85**:78–83.
10. LEATHEN, W. W. 1952. Microbiological studies of bituminous coal mine drainage. Commonwealth of Pennsylvania Dept. Health Industrial Fellowship 326B-6.
11. RAZZELL, W. E., AND P. C. TRUSSELL. 1963. Microbiological leaching of metallic sulfides. Appl. Microbiol. **11**:105–110.
12. RAZZELL, W. E., AND P. C. TRUSSELL. 1963. Isolation and properties of an iron-oxidizing *Thiobacillus*. J. Bacteriol. **85**:595–603.
13. SATO, M. 1960. Oxidation of sulfide ore bodies. II. Oxidation mechanisms of sulfide minerals at 25 C. Econ. Geol. **55**:1202–1231.
14. SILVERMAN, M. P., AND H. L. EHRLICH. 1964. Microbial formation and degradation of minerals. Advan. Appl. Microbiol. **6**:153–206.
15. SILVERMAN, M. P., AND D. G. LUNDGREN. 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77**:642–647.
16. SILVERMAN, M. P., AND D. G. LUNDGREN. 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. II. Manometric studies. J. Bacteriol. **78**:326–331.
17. SILVERMAN, M. P., M. H. ROGOFF, AND I. WENDER. 1961. Bacterial oxidation of pyritic materials in coal. Appl. Microbiol. **9**:491–496.
18. SILVERMAN, M. P., M. H. ROGOFF, AND I. WENDER. 1963. Removal of pyritic sulphur from coal by bacterial action. Fuel **42**:113–124.
19. STOKES, H. N. 1901. On pyrite and marcasite. U.S. Geol. Surv. Bull. 186.
20. TEMPLE, K. L., AND W. A. KOEHLER. 1954. Drainage from bituminous coal mines. West Va. Univ. Eng. Expt. Sta. Res. Bull. 25.
21. UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques. Burgess Publishing Co., Minneapolis.